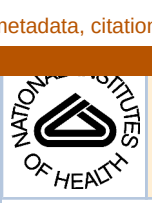

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PECAM-1 is necessary for flow-induced vascular remodeling

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Abstract

OBJECTIVE—Vascular remodeling is a physiological process that occurs in response to long-term changes in hemodynamic conditions, but may also contribute to the pathophysiology of intima-media thickening (IMT) and vascular disease. Shear stress detection by the endothelium is thought to be an important determinant of vascular remodeling. Previous work showed that Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a component of a mechanosensory complex that mediates endothelial cell (EC) responses to shear stress.

METHODS AND RESULTS—We tested the hypothesis that PECAM-1 contributes to vascular remodeling by analyzing the response to partial carotid artery ligation in PECAM-1 knockout mice and wild-type littermates. PECAM-1 deficiency resulted in impaired vascular remodeling and significantly reduced IMT in areas of low flow. Inward remodeling was associated with PECAM-1-dependent NFκB activation, surface adhesion molecule expression and leukocyte infiltration as well as Akt activation and vascular cell proliferation.

CONCLUSIONS—PECAM-1 plays a crucial role in the activation of the NFκB and Akt pathways and inflammatory cell accumulation during vascular remodeling and IMT. Elucidation of some of the signals that drive vascular remodeling represent pharmacologically tractable targets for the treatment of restenosis after balloon angioplasty or stent placement.

Keywords

hemodynamics; vascular remodeling; intima-media thickening; inflammation

Introduction

Vascular remodeling is the ability of the cells of the vessel wall to reorganize their cellular and extracellular components in response to a chronic stimulus [1]. For instance, increases in blood flow due to an arteriovenous shunt will increase vessel diameter; conversely, reductions in blood flow initiate a signaling cascade that leads to a reduction in the vascular lumen [2]. Vascular remodeling of the carotid artery, clinically defined as intima-media thickening (IMT), is an important predictive phenotype for human cardiovascular disease [3, 4].

An important stimulus for vascular remodeling is blood flow. As blood flows along a vessel, it creates shear stress on the vessel wall. Shear stress forces modulate endothelial structure and function. Due to normal laminar flow, shear stress is a critical factor in maintaining vascular

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homeostasis. On the other hand, disturbed shear stress is a determinant of localized atherosclerotic lesions at bifurcations and branch points. Many studies have described the correlations between local shear stress and plaque progression in human coronary arteries [5, 6]. Even in healthy subjects, carotid IMT is inversely related to carotid shear stress. Shear stress and vascular remodeling are also responsible for restenosis after balloon angioplasty or stent implantation [7, 8].

Vascular ECs are ideally positioned to serve as transducers, to relay hemodynamic and biochemical changes into molecular events in the other layers of the vascular wall [9]. EC surfaces are equipped with numerous mechanoreceptors capable of detecting and responding to shear stress, including caveolae, ion channels, integrins, receptor Tyr kinases, the apical glycocalyx, primary cilia, heterotrimeric G proteins, and intercellular junctions. In this context, we recently identified a mechanosensory complex comprised of PECAM-1, vascular endothelial cadherin (VE-cadherin), and vascular EC growth factor receptor-2 (VEGFR2) that mediates EC responses to shear stress [10]. Based on the significant role of PECAM-1 in transducing shear stress in ECs *in vitro*, we hypothesized that PECAM-1 plays an important role in vascular remodeling and IMT associated with changes in flow *in vivo*.

Methods

Cell culture, shear stress assays

PECAM-1$^{-/-}$ cells and cells reconstituted with murine full-length PECAM-1 were prepared as described[11]. Levels of PECAM-1 in reconstituted cells are similar to wild-type levels, and the identity of the ECs was confirmed by expression of VE-cadherin and uptake of Ac-LDL (not shown). For oscillatory flow, ECs were sheared at ± 6.5 Dyne/cm^2, 1 Hz (there was an average forward flow component of 0.4 dyne/cm^2 to allow for nutrient delivery).

Animals

PECAM-1$^{-/-}$ C57BL/6 mice were kindly provided by Dr. P. Newman (Blood Research Institute, Blood Center of Wisconsin, Milwaukee), bred in house and used in accordance with the guideline of the National Institute of Health and for the care and use of laboratory animals (approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill). Male PECAM-1$^{-/-}$ and age-matched littermates (PECAM-1$^{+/+}$)(12–14 weeks) were used for all experiments. All analyses were conducted by observers blinded to animal phenotype.

Experimental Protocol

Blood flow reduction in the left common carotid artery (LCA) and blood flow measurements were performed as previously described [12–14]. Briefly, C57Bl/6 mice were anesthetized with isoflurane (1.5%) and maintained at 37°C on heating pad. They received subsequent antibiotic (50 mg/kg cephazolin), and analgesic (10 mg/kg im Pentazocine) after wound closure. Sterile technique was used to isolate ~0.5-mm lengths of the left external carotid artery distal to the thyroid artery and the left internal carotid/occipital artery pair, followed by ligation with 7-0 suture. Partial blood flow (5–10% within 3 weeks after surgery) was maintained through the thyroid branch. The sham procedure consisted of vessel isolation and ligature placement without ligation. To measure blood flow, 0.7V series transonic flow probe was used according to the manufacturer's instructions (http://www.transonic.com/workbook.shtml).

Morphometry

At 24-hr, 5-day or 3-week after the surgical procedure, the vasculature was fixed by transcardial perfusion at 100 mmHg with 25 ml sodium nitroprusside in 0.1mM PBS followed by 100 ml

of 4% paraformaldehyde. The trachea, left and right carotid arteries, and surrounding tissue were removed *en bloc*, and fixed for 24 h in 4% paraformaldehyde. The central 5mm section was embedded in paraffin. Sections (5 μm) were cut every 300 μm through the central-most 2.5-mm length, and eight sections were mounted to a slide; additional slides were prepared in the same way by serially sectioning at these intervals along the vessel. For morphometric analyses, slides from the 3-week time point were stained with Masson's trichrome for morphometry or hematoxylin-eosin for cell density and morphology. Images were taken with a Zeiss inverted microscope. The circumferences of lumen (CL), internal and external elastic lamina of common carotid artery (IEL, EEL) were measured from the images using NIH Image J package. Calculations were performed as follows:

$$\text{Lumen area}=\frac{\text{LC}^2}{4\pi},\ \text{intima}-\text{media thickness}=\frac{\text{EEL}-\text{LC}}{2\pi}$$

Immunohistochemistry

Immunohistochemistry for paraffin embedded cross sections was described previously [13]. For *en face* preparations, the common carotid artery was perfusion fixed and dissected out under dissection microscope. The common carotid arteries were cut longitudinally and pinned flat with endothelium facing up onto a Surperfrost/Plus glass slide (Fisher Scientific, Pittsburgh, PA). We used antibodies to NFκB (1:100, BD Pharmingen, San Diego, CA), phosphorylated NFκB S536 (1:100, Cell Signaling), ICAM-1 (1:100, Santa Cruz, CA), VCAM-1 (1:100, Santa Cruz), phosphorylated Akt pS473 (1:100, Cell Signaling), CD45 (1:50, BD Pharmingen). Antigen retrieval was performed for cross sections with Retrogen (BD Pharmingen), except for NFκB and ICAM-1 antibodies. For Avidin—Biotin—peroxidase Complex (ABC) staining, primary antibodies were incubated at 4°C overnight, followed by 60 minutes for secondary antibody incubation at room temperature, and 30 minutes for ABC complex (Vector Elite). 3,3' diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA) was used to visualize the peroxidase-binding sites, followed by Mayor modified hematoxylin staining. For *en face*, slides were permeabilized with 0.3% Triton-PBS for 40 minutes, and blocked with 10% fetal bovine serum for 60 minutes. After overnight incubation of primary antibodies at 4°C, the slides were incubated with FITC-labeled secondary antibodies for 60 minutes. *En face* preparations were evaluated with a Leica confocal microscope. For quantitation studies, 5 random fields and <100 cells were counted.

Luciferase activity assays

Luciferase activity assays were performed as described [15] using a vector (1.0μg) containing the PDGF-A-chain shear stress response element (PDGF-A/SSRE) regulating the expression of firefly luciferase, together with *Renilla* luciferase, under the control of a minimal promoter[16]. Transfections were performed using Lipofectamine LTX Reagent (Invitrogen).

Leukocyte adhesion assay

These assays were performed as previously described [17]. To quantify adherent cells, five random fields were counted for each assay.

Statistical Analysis

Results are described as mean ± SEM. Statistical tests were performed with Microsoft Excel analysis package, using Student t test for 2 groups or one-way ANOVA followed by multiple comparisons with Honest Significance Difference (HSD) test. The level of $P<0.05$ was considered significant.

Results

Vascular Remodeling After Flow Alteration in PECAM-1$^{-/-}$ mice

In order to assess how changes in blood flow and attendant shear stress affect arterial remodeling *in vivo*, we used the partial carotid ligation model, and examined flow-dependent remodeling in the common carotid arteries of PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ mice. This model limits thrombosis and EC denudation and is thus physiologically relevant when studying cardiovascular disease [12]. Importantly, carotid flows were comparable in PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ mice before and after ligation (Fig S1). While there were no differences in sham PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ vessels, we did observe dramatic differences in the LCAs of ligated animals (Fig. 1). Wild-type ligated LCAs had reduced lumen diameter and a dramatic increase in intima-media thickness; in contrast, the LCA IMT of ligated PECAM-1$^{-/-}$ mice was dramatically thinner. Increased flow in the RCA caused a small compensatory increase in lumen area in the PECAM-1$^{+/+}$ but not in the PECAM-1$^{-/-}$ vessels (Fig. 1). Thus, based on the fact that PECAM-1 deficiency resulted in a striking decrease in intima-media and adventitia thickening induced by ligation, we hypothesized that PECAM-1-dependent signaling might be important during vascular remodeling.

Proliferation in PECAM-1$^{-/-}$ Mice

Several studies have shown that proliferation is a driving force for remodeling in response to low flow [12, 18, 19], while apoptosis is also thought to have a central role in IMT [19]. Akt plays a particularly prominent part in signaling networks that modulate cellular proliferation, apoptosis and survival. To gain further insight into mechanisms by which PECAM-1 alters IMT, we evaluated phosphorylation of Akt, apoptosis and proliferation in the LCA. LCAs from PECAM-1$^{+/+}$ and PECAM-1$^{-/-}$ mice were fixed, excised and examined *en face*. We observed very low levels of pAkt in the ECs of LCAs from shams (Fig. 2A; insets). pAkt levels were significantly upregulated in WT LCAs; however, there was a dramatic decrease in pAkt positive staining in the ECs of PECAM-1$^{-/-}$ (Fig. 2A). To assess proliferation, cross-sections of the carotid arteries were immunostained for PCNA with hematoxylin counterstain to obtain area and nuclear cell density of media and adventitia. In the LCA from sham operated animals from both genotypes we found no cell proliferation. As expected, PECAM-1$^{+/+}$ animals showed increased proliferation in all three layers in the LCA; in contrast, proliferation was greatly reduced in the PECAM-1 $^{-/-}$ mice after ligation, especially in the intima layer (Fig. 2B). We also assayed apoptosis in both genotypes by staining for cleaved caspase-3. Despite the role for PECAM-1 in apoptosis [20, 21], we did not observe differences in apoptosis (not shown). We therefore conclude that PECAM-1 signaling is required for flow-induced Akt activation and proliferation observed in areas of low flow.

NFκB Pathway in Intima-Media in PECAM-1−/− Mice

Increased expression of a wide array of inflammatory genes, many of which contain NFκB consensus sites within their promoters, is a prominent feature of neointima formation [22]. In addition, the NFκB signal transduction pathway is regulated by blood flow and is primed for activation in regions exposed to disturbed flow [23]. The impaired IMT and vascular remodeling in PECAM-1$^{-/-}$ carotids raises the possibility that PECAM-1 participates in the regulation of the inflammatory response elicited by ligation. Because proximal to the site of stenosis (or ligation), disturbed shear stress prevails and adversely affects the biology of the arterial wall [24], we used an *in vitro* system to apply disturbed shear stress to ECs that express (PE-RC) or lack (PE-KO) PECAM-1. PE-RC and PE-KO ECs were exposed to oscillatory flow to allow sustained activation of NFκB, which was assessed by either staining with the phospho-p65 Ab (Fig.S1), or measuring transcriptional activation of NFκB using a luciferase reporter (Fig. 3A). Both assays showed that ECs expressing PECAM-1 are able to activate NFκB, in contrast to cells lacking PECAM-1.

Because PECAM-1 is required for NFκB activation in response to oscillatory flow *in vitro* (Fig. 3A), we asked whether the NFκB pathway might explain differences in PECAM-$1^{-/-}$ and PECAM-$1^{+/+}$ carotid remodeling. As shown in Fig. 3B, although ECs from PECAM-$1^{+/+}$ LCAs showed nuclear accumulation for the p65 subunit of NFκB, ECs from PECAM-$1^{-/-}$ mice showed cytoplasmic localization for NFκB. Importantly, activation of NFκB in PECAM-$1^{+/+}$ LCAs is an early event in response to ligation, it was observed as early as 2h and reached a maximum at 24h post ligation.

NFκB dimers bind to a shear stress responsive element found in the promoter of several atherogenic genes, including ICAM-1 and VCAM-1, which regulate monocyte recruitment [25]. To test if PECAM-1-dependent NFκB activation translates to altered gene expression, we assayed expression of ICAM-1 and VCAM-1 in response to oscillatory flow. Flow cytometric analysis and immunofluorescence surface staining showed that cells expressing PECAM-1 upregulated both ICAM-1 and VCAM-1. In contrast, PECAM-1 null ECs were unresponsive to flow (Fig. S3). Thus, PECAM-1 expression contributes to the pro-inflammatory phenotype of the endothelium induced by disturbed shear stress.

We then assayed upregulation of these NFκB target genes during flow-induced IMT. We did not observe any differences in VCAM-1 or ICAM-1 staining between sham-operated PECAM-$1^{+/+}$ and PECAM-$1^{-/-}$ mice. However, expression of VCAM-1 and ICAM-1 in ligated LCAs was much lower in PECAM-$1^{-/-}$ compared to PECAM-$1^{+/+}$ animals (Fig. 4). We also analyzed expression of ICAM-1 and VCAM-1 in ECs using cross-sections of the carotid (Fig.S2). Interestingly, the NFκB activation described above was temporally followed by CAM upregulation, which was seen as early as 24–48h and lasted for up to 2 weeks after ligation in PECAM-$1^{+/+}$ LCAs (not shown). Taken together, these results demonstrate that lack of PECAM-1 signaling leads to a disruption of the NFκB pathway, thereby regulating vascular remodeling and IMT.

Role of PECAM-1 in flow-induced Inflammation

Upregulation of CAMs is a fundamental prerequisite for the attraction and adhesion of monocytes. This inflammatory response is also important during vascular remodeling [12]. Because disturbed flow induces monocyte adhesion both *in vivo* and *in vitro* by upregulating CAM expression, we hypothesized that disruption of the NFκB pathway in PECAM-$1^{-/-}$ mice affects monocyte adhesion in response to disturbed flow and during vascular remodeling. Exposure of PECAM-1-expressing ECs to disturbed flow significantly increased monocyte adhesion (Fig. 5A). In contrast, PECAM-1 null ECs could not support monocyte adhesion in response to disturbed flow. Importantly, treatment with TNF-α increased monocyte adhesion independently of PECAM-1 expression, suggesting that the inflammation defect is due to disrupted mechanosignaling rather than due to a direct role for PECAM-1 in transmigration in this system (Fig. 5A). To test whether loss of PECAM-1 signaling affected flow-induced inflammation during vascular remodeling, we used immunohistochemistry for CD45. We found much greater CD45 positive cell accumulation in LCAs of wild-type mice compared to matched areas of PECAM-$1^{-/-}$ mice (Fig. 5B). As was previously reported for C57Bl/6 mice, inflammatory cells were not detected in LCA from sham operated animals in both genotypes. These results therefore suggest that PECAM-1 regulates CAM expression and monocyte adhesion in response changes in flow *in vitro* and *in vivo*.

Discussion

Shear stress is a biomechanical force generated by fluid flow on the surface of the endothelium and plays a major role in vascular physiology and disease. However, the mechanisms by which ECs sense this mechanical stimulus and transform it into intracellular biochemical signals that change vessel structure and function have not been fully characterized *in vivo*. PECAM-1 is

thought to be involved in flow mechanosensing or transduction, based on changes in its phosphorylation with altered pressure and flow [26–28] and *in vitro* experiments showing PECAM-1-dependent activation of flow-mediated intracellular signaling pathways [26, 29–32]. More recently, researchers used *ex vivo* approaches: isolated skeletal muscle arterioles of PECAM-1$^{-/-}$ mice to show reduced NO-mediated dilation in response to high temporal gradients of wall shear stress [33], as well as a requirement for PECAM-1 for flow-mediated dilation in the mouse coronary circulation [34]. During preparation of this manuscript, two studies reported a role for PECAM-1 in regulating atherosclerosis[35, 36]. Our findings support and extend these studies by showing that PECAM-1 is also important for vascular remodeling. Although it is important to understand the contribution of PECAM-1 in atherosclerosis, atherosclerosis is a multi-factorial disease influenced by both systemic and mechanical factors. Our findings provide the first demonstration that PECAM-1 is required for flow-mediated remodeling and IMT. We show that activation of NFκB; downstream upregulation of ICAM-1; and inflammatory cell accumulation in the low flow areas do not occur in the PECAM-1$^{-/-}$ mice. Additionally, PECAM-1$^{-/-}$ LCAs had significantly impaired Akt activation and proliferation. As a consequence, PECAM-1 deficient vessels are protected from hypertrophic remodeling seen in the low flow areas. Interestingly, the dramatically reduced IMT in PECAM-1$^{-/-}$ LCAs is similar to that seen in mice deficient in p105, the precursor of p50, one of the components of NFκB [22]. These data support the importance of the PECAM-1/ NFκB axis in vascular remodeling Importantly, although PECAM-1 is important in transendothelial migration of monocytes and neutrophils[37, 38], its role in transendothelial migration depends on the mouse strain, as C57BL/6 mice showed no discernible effects in two models of inflammation[39]. Because we investigated flow-mediated remodeling in mice on a C57BL/6 background, it is unlikely that PECAM-1 absence significantly curbed remodeling by inhibiting transmigration. However, we cannot rule out the contribution of the inflammatory role of PECAM-1 to the observed phenotype. PECAM-1 may also regulate vascular remodeling by mechanisms not investigated here. PECAM-1$^{-/-}$ mice have increased bleeding times[40] which might affect vascular remodeling and IMT. PECAM-1 has also been shown to regulate eNOS activity[32–34], which in turn affects remodeling[41].

Flow-dependent vascular remodeling involves multiple genes, cell types and processes [42]; indeed, recent studies using inbred mouse or rat strains emphasized the role of genetic factors in remodeling and IMT [18, 43]. Amongst candidate mediators that have been extensively studied in mechanotransduction *in vitro* and remodeling *in vivo* are caveolae[44, 45]. Interestingly, flow-dependent remodeling in Caveolin-1 null carotids phenocopies the abnormal remodeling in eNOS-deficient mice[41], thus underscoring the importance of both in vascular remodeling. Components of integrin signaling, such as β1 integrin, focal adhesion kinase and vimentin are also important for vascular remodeling [46–48]. In addition, generation of reactive oxygen species (ROS) plays a central role in mechanotransduction and vascular remodeling [49, 50]. Finally, the role of inflammation and white blood cells in vascular remodeling has become increasingly apparent [19, 51].

Shear stress detection by the endothelium has been proposed to be the primary mediator of flow-induced remodeling [2]. The elucidation of a previously unrecognized role for PECAM-1 signaling in regulating vascular remodeling and the elucidation of some of the signals that drive remodeling considerably expand our understanding of the repertoire of molecular regulators controlling this process. Therefore, further characterization of PECAM-1 signaling and its pharmacological modulation might lead to novel therapeutic strategies for the treatment of restenosis after balloon angioplasty or stent placement.

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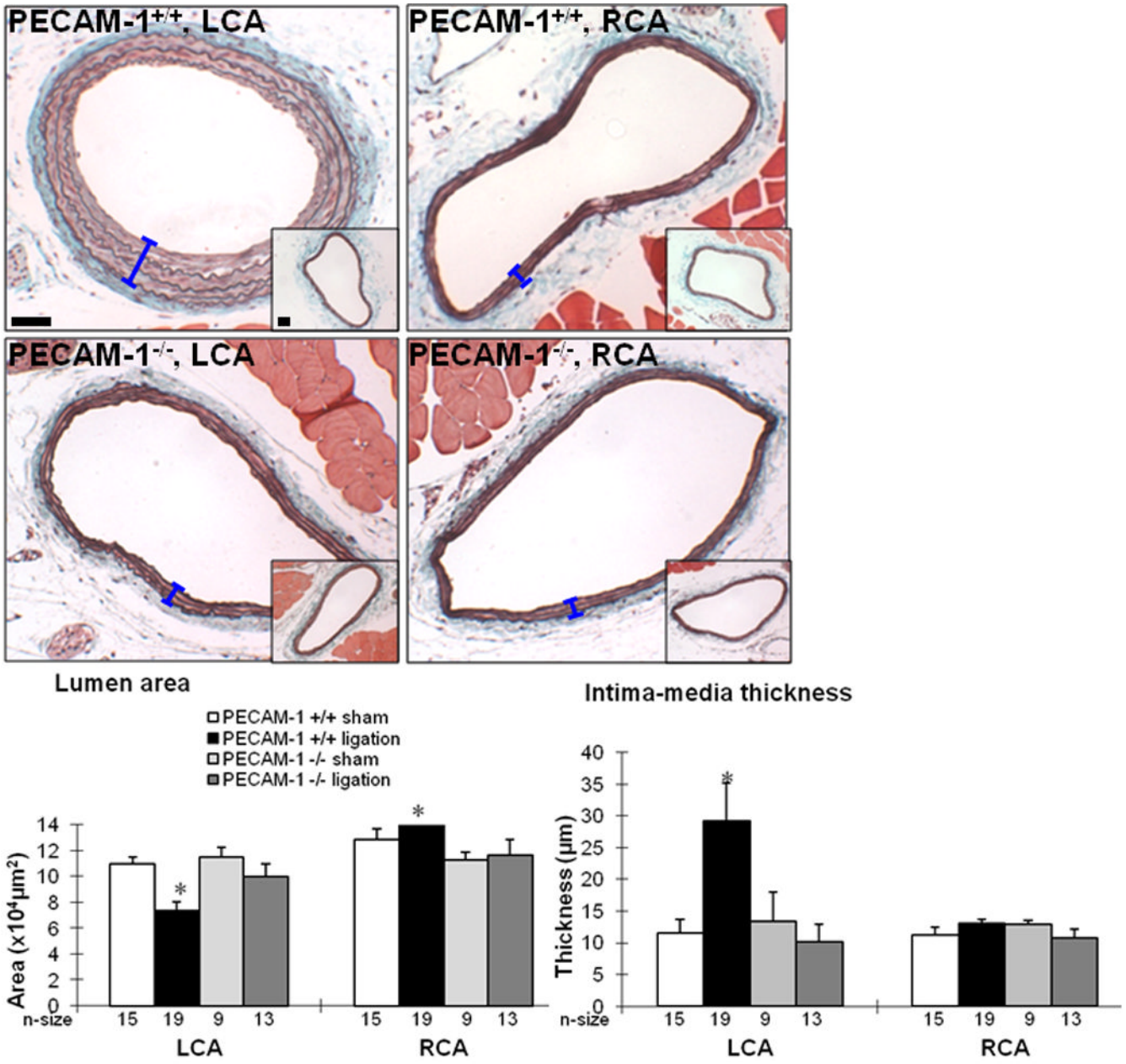

Figure 1. PECAM-1 is necessary for flow-induced remodeling *in vivo*

(**A**). Cross-sections of carotid arteries 3 weeks after ligation. Sham LCAs shown as insets. Bracket shows IMT. Bar, 50μm. (**B**). Morphometric analysis of carotid artery lumen area and IMT. * *P* <0.05 vs sham.

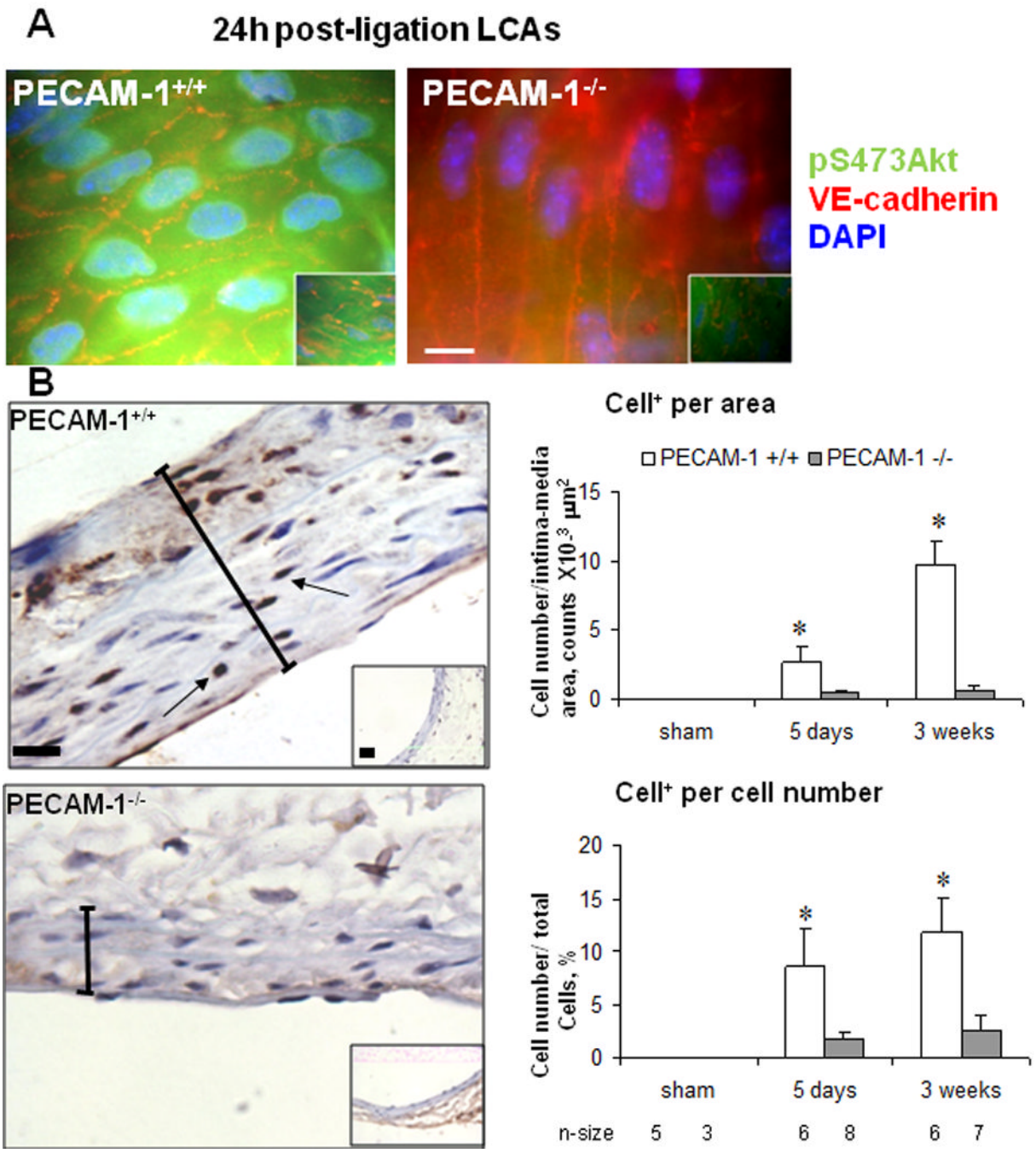

Figure 2. PECAM-1 is necessary for proliferation during vascular remodeling

(A) *En face* LCAs stained for pS473Akt, VE-cadherin and DAPI. Bar, 10µm. **(B)** Cross-sections of LCAs stained for PCNA and counter stained with hematoxylin. PCNA positive cells were normalized per area, or per total cells. * P <0.05 vs. Bar=20µm

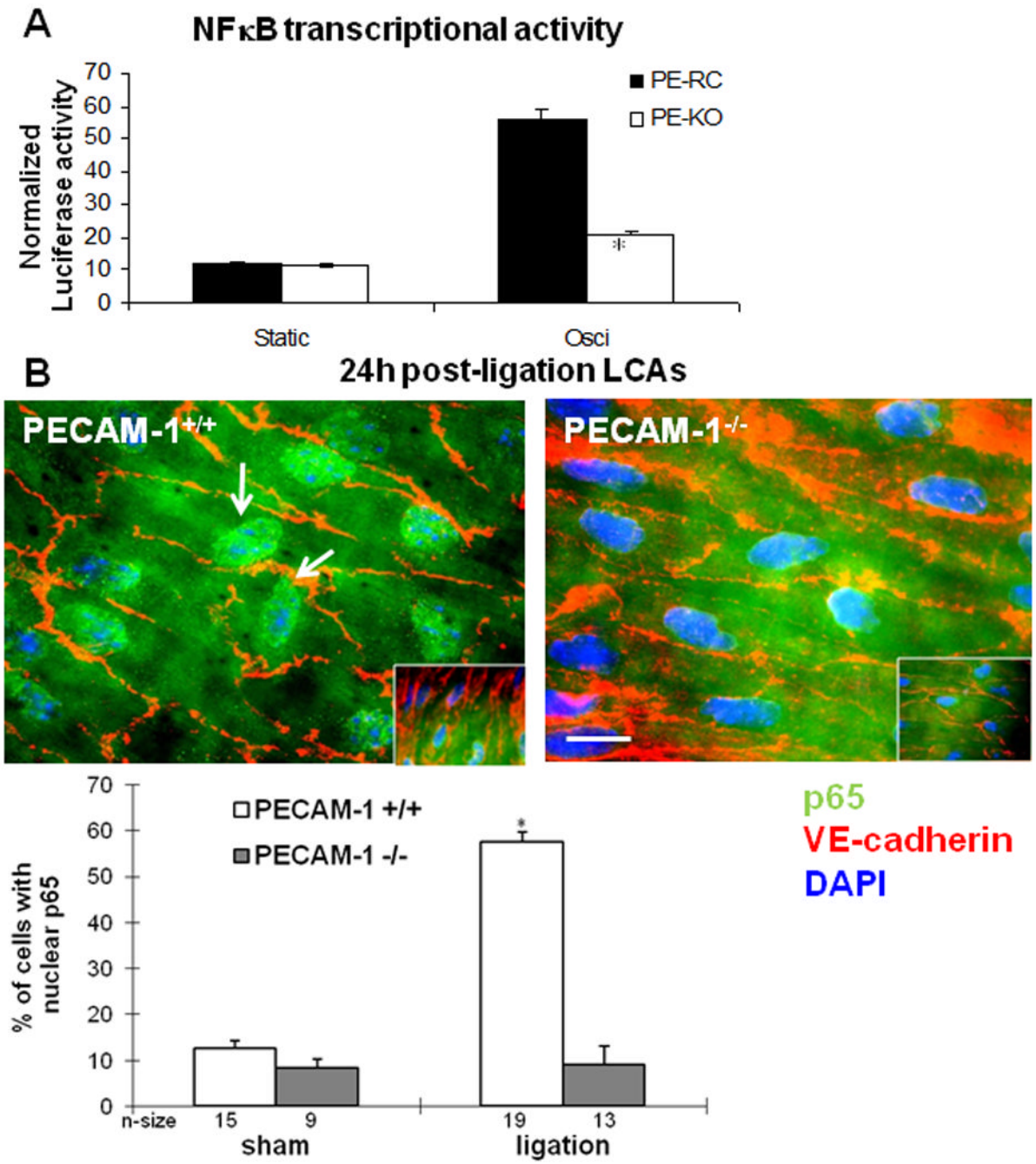

Figure 3. PECAM-1 is necessary for flow-induced NFκB activation in response to flow and during vascular remodeling

(**A**). PECAM-1 knockout (PE-KO) or reconstituted (PE-RC) ECs were exposed to oscillatory flow and assayed for NFκB transcriptional activity (* $p < 0.05$). **(B)** *En face* LCAs stained for p65, VE-cadherin and Dapi.. Bar, 10μm.

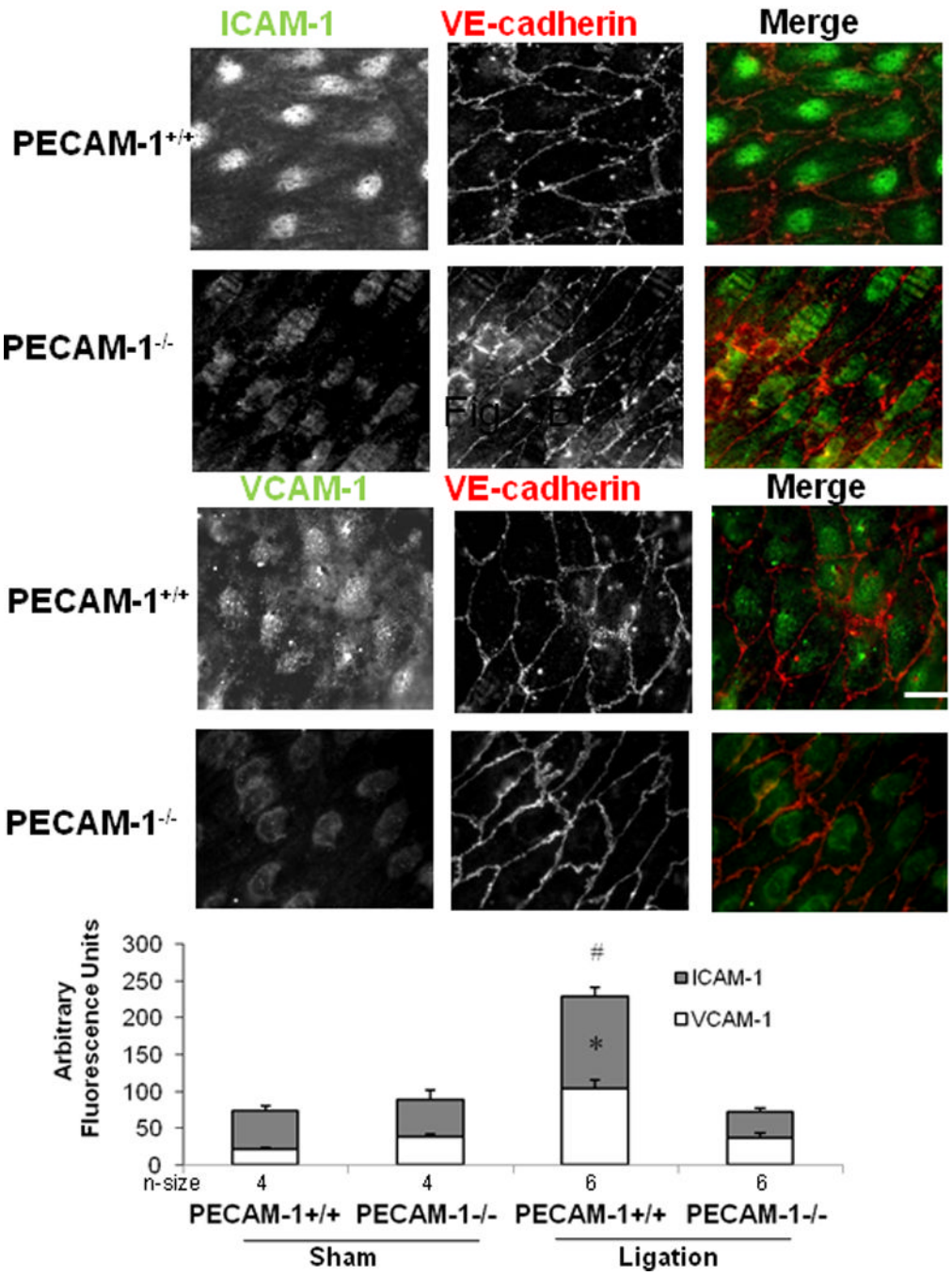

Figure 4. PECAM-1 is necessary for inflammatory gene expression during vascular remodeling
En face LCAs stained for ICAM-1 and VE-cadherin. # *P* <0.05 vs sham. Bar, 60μm

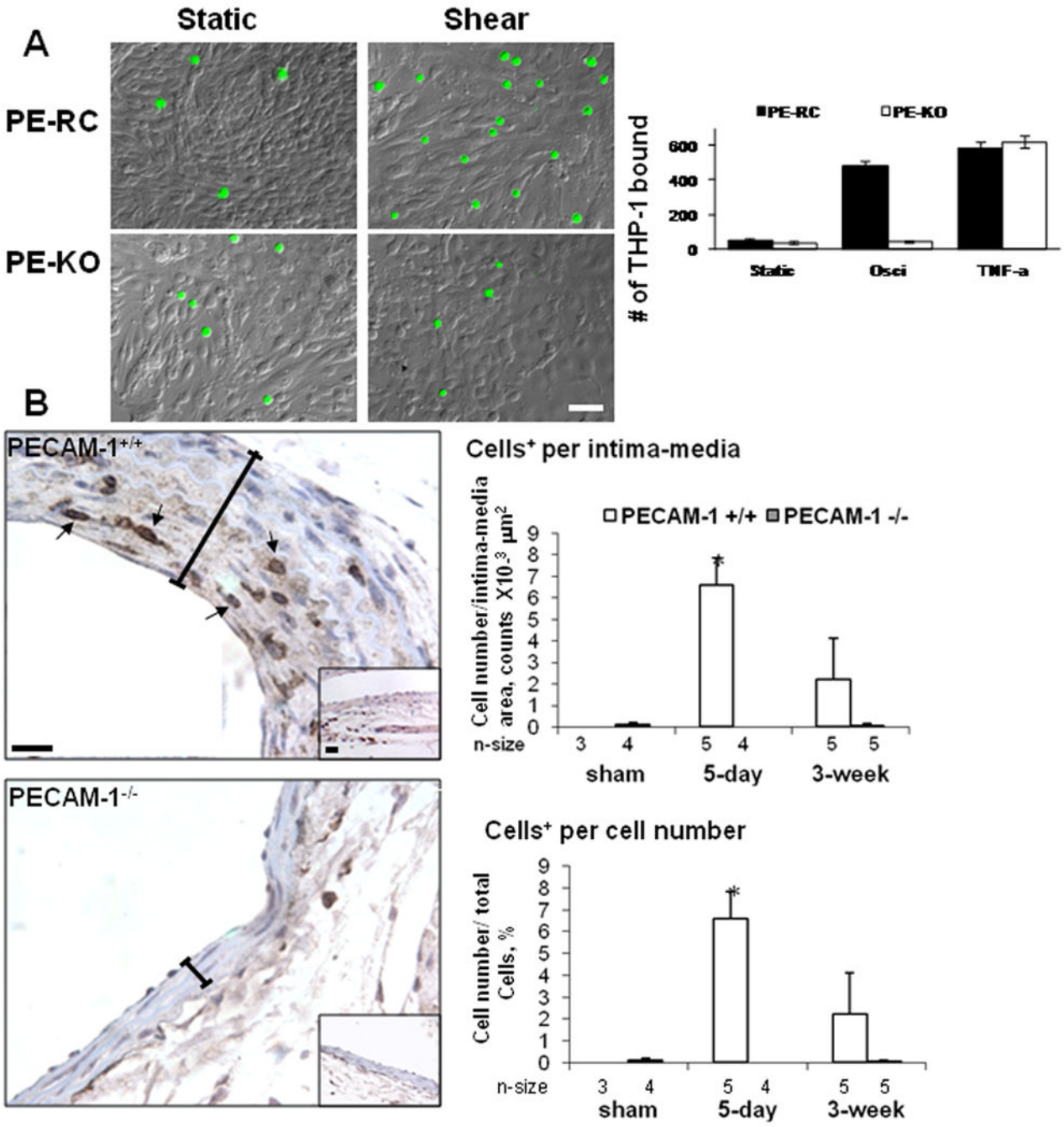

Figure 5. PECAM-1 promotes leukocyte accumulation during vascular remodeling

(A) PE-RC or PE-KO cells were exposed to oscillatory flow or TNF-α and monocyte cell adhesion was assayed. Bar, 50μm. Quantitation of monocyte binding assays in also shown. (* $p < 0.05$). **(B)** Cross-sections of LCAs were stained with CD45 antibody and counter stained with hematoxylin. * $P < 0.05$ vs sham. Bar, 20μm.